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Functional consequences of single amino acid substitutions in calmodulin-activated adenylate cyclase of *Bordetella pertussis*

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Calmodulin-activated adenylate cyclase of *Bordetella pertussis* and *Bacillus anthracis* are two cognate bacterial toxins. Three short regions of 13-24 amino acid residues in these proteins exhibit between 66 and 80% identity. Site-directed mutagenesis of four residues in *B.pertussis* adenylate cyclase situated in the second (Asp188, Asp190) and third (His298, Glu301) segments of identity were accompanied by important decrease, or total loss, of enzyme activity. The calmodulin-binding properties of mutated proteins showed no important differences when compared to the wild-type enzyme. Apart from the loss of enzymatic activity, the most important change accompanying replacement of Asp188 by other amino acids was a dramatic decrease in binding of 3'-anthraniloyl-2'-deoxyadenosine 5'-triphosphate, a fluorescent analogue of ATP. From these results we concluded that the two neighbouring aspartic acid residues in *B.pertussis* adenylate cyclase, conserved in many other ATP-utilizing enzymes, are essential for binding the Mg^{2+}-nucleotide complex, and for subsequent catalysis. Replacement of His298 and Glu301 by other amino acid residues affected the nucleotide-binding properties of adenylate cyclase to a lesser degree suggesting that they might be important in the mechanism of enzyme activation by calmodulin, rather than being involved directly in catalysis.

Key words: ATP-binding properties/*B.pertussis* adenylate cyclase/catalytic mechanism/site-directed mutagenesis

Introduction

ATP-dependent enzymes are among the most common catalysts in the living kingdom. In spite of this ubiquity, the actual nature of the ATP-binding site and mechanisms of various catalytic processes involving the nucleotide are seldom known in detail. Adenylate cyclase, which catalyses the reversible cyclizing reaction ATP $\longleftrightarrow$ cAMP + PP_i + H^+, plays an important regulatory role both in prokaryotes and eukaryotes (Ullmann and Danchin, 1983; Gancedo *et al.*, 1985). In recent years, several adenylate cyclases, including two cognate bacterial toxins, i.e. adenylate cyclase from *B.pertussis* and *B.anthracis* have been cloned, sequenced, and overproduced in our laboratories (Aiba *et al.*, 1984; Masson *et al.*, 1986; Escuyer *et al.*, 1988; Glaser *et al.*, 1988a; Beuve *et al.*, 1990). The latter two enzymes share several common properties, such as activation by calmodulin (CaM), high specific activity, and invasiveness i.e. the capacity to enter eukaryotic cells provoking an uncontrolled increase in cAMP levels and in cell death (Confer and Eaton, 1982; Leppla, 1982; Rogel *et al.*, 1988; Hewlett *et al.*, 1989; Masure and Storm, 1989; Bellalou *et al.*, 1990). The difference in size between adenylate cyclase from *B.pertussis* (1706 amino acid residues) and *B.anthracis* (800 amino acid residues) is most probably due to the fact that both proteins are endowed with functions other than CaM-activated cyclization of ATP (Leppla, 1984; Glaser *et al.*, 1988b; Cataldi *et al.*, 1990). In fact, truncated *cya* genes from *B.pertussis* and *B.anthracis* expressed in *E.coli* yielded fully active enzymes of only 399 (*B.pertussis*) and 542 (*B. anthracis*) amino acid residues, respectively (Gilles *et al.*, 1990; Labruyère *et al.*, 1990). Despite little sequence similarity, three short regions of 13-24 amino acid residues in these proteins exhibited between 66% and 80% identity (Figure 1), suggesting that they might be implicated in some common and important catalytic or regulatory functions. The first region contains amino acids resembling the consensus sequence GXXXXGKT(S) present in many nucleotide-binding proteins, and it has previously been shown that this region was indeed important for catalysis (Au *et al.*, 1989; Glaser *et al.*, 1989; Xia and Storm, 1990; Labruyère *et al.*, 1991). The two other regions (situated between residues 184-196 and 294-314, respectively) bracket a helical structure in *B.pertussis* adenylate cyclase situated around Trp242 that has been shown to interact with CaM (Glaser *et al.*, 1989; Ladant *et al.*, 1989). Having in mind the seemingly 'modular' construct of *B.pertussis* adenylate cyclase (Ladant *et al.*, 1989; Gilles *et al.*, 1990) where the N-terminal domain (situated within amino acid residues 1-224) corresponds mainly to

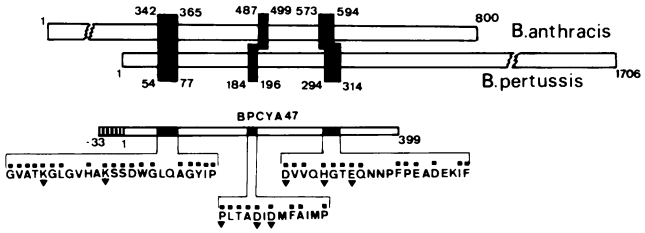

Fig. 1. Schematic representation of *B.pertussis* and *B.anthracis* adenylate cyclases, with indication of sequences exhibiting a high degree of homology (black boxes). BPCYA47 is a truncated form of *B.pertussis* adenylate cyclase expressed in *E.coli*. Hatched box labelled -33 to 1 represents the foreign sequence encoded by the vector. In each sequence (amino acids being given by the one-letter code) within residues 54-77, 184-196 and 294-314 in *B.pertussis* adenylate cyclase squares indicate identical residues in *B.anthracis* enzyme. Triangles situated under amino acids indicate residues submitted to site-directed mutagenesis.

the catalytic site, while the C-terminal segment (situated within residues 225–399) belongs to the CaM-binding domain, we hypothesized that the sequence 184–196 might contain amino acid residues involved in nucleotide-binding or/and catalysis, whereas the sequence 294–314 should be involved in binding or/and activation of adenylate cyclase by CaM.

In this paper we show that site-directed mutagenesis of four residues situated in the second and in the third segments of identity of the two bacterial toxins (Asp188, Asp190, His298 and Glu301) is indeed accompanied by an important decrease, or even total loss of enzyme activity. To understand the functional role of these residues in the bacterial enzyme, modified proteins overexpressed in *E.coli* were purified and analysed for their ligand-binding properties using fluorescent derivatives of CaM and ATP.

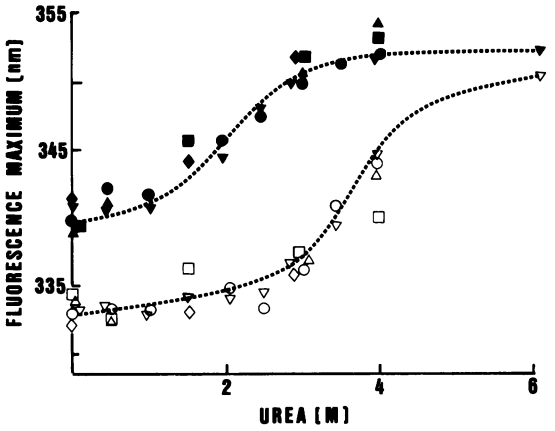

Fig. 2. Effect of different concentrations of urea on the fluorescence emission maximum of *B.pertussis* adenylate cyclase alone (closed symbols) or in the presence of CaM (open symbols). (▽, ▼) wild-type adenylate cyclase; (○, ●) D188N mutant; (□, ■) D188Y mutant; (△, ▲) D190Y mutant; (◇, ◆) H298R mutant.

Results

Purification and analysis for intrinsic fluorescence properties of BPCYA47 and of its modified forms

Truncated adenylate cyclase of *B.pertussis* expressed in *E.coli* was purified by CaM-agarose chromatography and analysed for intrinsic fluorescent properties. Although the fluorescence bands of the two Trp residues (W69 and W242) were largely overlapping, the spectrum of adenylate cyclase underwent significant changes (a shift of the maximum and narrowing or broadening of bands), in the presence of CaM, alone or in association with urea (Gilles *et al.*, 1990). These modifications, reflecting local structural changes in the protein upon interaction with ligand or denaturing agents, were used to test CaM-binding properties and stability of the enzyme molecule in which different amino acid residues were substituted by site-directed mutagenesis. All modified forms of *B.pertussis* adenylate cyclase exhibited fluorescent properties similar to those of the parent protein. Moreover, the shift in the fluorescence maximum in the presence of various concentrations of urea did not differ essentially from that of wild-type protein (Figure 2). H298L and E301K forms of adenylate cyclase showed a slightly increased sensitivity towards denaturation by urea, whereas the D190N modified variant of BPCYA47 had a tendency to form insoluble aggregates upon storage.

Table I. Amino acid substitutions yielding weakly active or inactive forms of *B.pertussis* adenylate cyclase[a]

Amino acid substituted[b]	Codon alteration	Sequence of mutagenic oligonucleotide[c]	% of wild-type activity in crude extract[d]
D188E	GAT→GAA	TGACGGCGGAAATCGACAT	0.1
D188N	GAT→AAT	CTGACGGCGAATATCGACA	<0.01
D188Y	GAT→TAT	CTGACGGCGTATATCGACA	n.d.[e]
D188H	GAT→CAT	CTGACGGCGCATATCGACA	n.d.
D190N	GAC→AAC	GCGGATATCAACATGTTC	<0.01
D190Y	GAC→TAC	GCGGATATCTACATGTTC	n.d.
D190H	GAC→CAC	GCGGATATCCACATGTTC	n.d.
H298R	CAT→CGT	TGGTCCAGCGTGGCACTGA	0.05
H298P	CAT→CCT	TGGTCCAGCCTGGCACTGA	0.03
H298L	CAT→CTT	TGGTCCAGCTTGGCACTGA	0.03
E301Q	GAG→CAG	CATGGCACTCAGCAGAACAA	20.0
E301K	GAG→AAG	CATGGCACTAAGCAGAACAA	0.02

[a]Several other mutations yielding adenylate cyclase species with activities close to the wild-type enzyme (P184L, P184R, P184Q, D294N, or D294Y) were not included in this table.
[b]Numbers refer to the position of amino acid in the primary structure of *B.pertussis* adenylate cyclase, and letters indicate the amino acid in the wild-type protein (left side) and the modified protein (right side), respectively.
[c]Underlined nucleotides correspond to the mismatches introduced.
[d]Bacteria sonicated in 50 mM Tris–HCl (pH 8) were centrifuged at 10000 *g* for 10 min: the sediment extracted with 8 M urea in the same buffer was used in enzymatic assays. Adenylate cyclase activity was normalized by dividing measured activity by the absorbance of the protein band identified in crude extract after SDS–PAGE and Coomassie blue staining. The activity of wild-type adenylate cyclase in crude extracts (about 280 U/mg of protein) was considered as 100%.
[e]n.d., not detected.

Single amino acid substitutions yielding weakly active/inactive forms of B.pertussis adenylate cyclase

From the six amino acid residues targeted as being important in expressing efficient catalysis in *B.pertussis* adenylate cyclase, four proved to be critical (Asp188, Asp190, His298 and Glu301). The residual activity of the modified proteins differed little as a function of the residue substituted. This seems to indicate that the four above-mentioned amino acids possess highly specialized roles in the function of bacterial cyclase. Even conservative replacements of Asp188 by Glu or Asn resulted in a decrease of activity by three and more than four orders of magnitude, respectively (Table I). A single exception was noticed: the E301Q modified variant of adenylate cyclase still possessed up to 20% of the activity of BPCYA47, whereas the residual activity of the E301K variant was ~1000 times lower. It was somewhat surprising that substitutions made at Pro184 (which is part of a conserved sequence in other phosphotransferases) or at Asp294 did not affect significantly the catalytic activity of modified proteins.

Interaction of CaM with the modified forms of adenylate cyclase

Adenylate cyclase of *B.pertussis* has a very high affinity for CaM (K_d = 0.2 nM) which could be determined accurately only from kinetic experiments. The affinity for CaM of inactive forms of *B.pertussis* adenylate cyclase (K'_d) was determined indirectly by competitive binding experiments i.e. from the inhibition of wild-type adenylate cyclase activity in the presence of fixed amounts of modified proteins (see Appendix). The concentrations of both wild-type (E) and modified (P) adenylate cyclases were in large excess (>10-fold) over CaM. Under these conditions the concentration of free, unliganded CaM was negligible and the ratio of catalytic activity in the presence and absence of inactive adenylate cyclase (x) was used to calculate K_d according to the formula:

$$K'_d = [P]\frac{K'_d}{[E]} \times \frac{x}{(1-x)}$$

Using this method we determined that the K_d for CaM of the D188N, H298R and E301K forms of adenylate cyclase were three to four times higher than that of the wild-type enzyme. On the other hand, D188Y, D190Y and H298L mutants exhibited 10–16-fold higher K_d values for CaM (Table II).

Binding of Ant-dATP to the modified forms of adenylate cyclase

Ant-dATP, a fluorescent analogue of ATP, was shown to bind with high affinity to the catalytic site of *B.pertussis* adenylate cyclase (Sarfati *et al.*, 1990). Binding of Ant-dATP to adenylate cyclase occurs preferentially in the presence of CaM and is accompanied by enhancement of the fluorescence yield of the nucleotide. We were interested to know if different substitutions affecting Asp188, Asp190, His298 and Glu301, in addition altering catalysis, also affected binding of nucleotide to adenylate cyclase. With a single exception, all of the modified forms of adenylate cyclase enhanced the fluorescence of Ant-dATP but to a much lesser degree than the wild-type protein. This reflects severe decrease in the affinity of nucleotide analog for the catalytic site. Equilibrium dialysis experiments using radioactive Ant-dATP confirmed and extended the conclusions obtained from fluorescence measurements. While CaM-complexed CYA47 binds the nucleotide analog in a stoichiometric ratio close to 1:1 (apparent K_d 8 μM), D188Y mutant exhibited very low nucleotide-binding properties ($K_d \geq 300$ μM). The other modified form of adenylate cyclase had K_d values for Ant-dATP between 6 and 8 times higher than that of wild-type protein (Table III). An interesting exception was the H298L modified adenylate cyclase. Whereas equilibrium dialysis experiments indicated that Ant-dATP binds to the enzyme, fluorescence analysis gave negative results. An explanation for this discrepancy is that subtle conformational changes occurring at the active site of CYA47 as the result of the H298L substitution would result in a serious decrease in the quantum yield of enzyme-bound Ant-dATP.

Discussion

Within recent years, site-directed mutagenesis along with X-ray crystallography has become a powerful technique in proving reaction mechanisms or substrate specificity of enzymes (Leatherbarrow and Fersht, 1986; Shaw, 1987; Scrutton *et al.*, 1990). In the absence of three-dimensional structural data, the prediction of amino acid residues belonging to the active site of enzymes is based mainly on sequence comparisons with related proteins. Although possibly fortuitous, similarities might reflect conservation of structural motifs in proteins carrying related functions. Amino acid residues potentially involved in binding of nucleotide or/and catalysis in *B.pertussis* adenylate cyclase were identified after comparison with another CaM-activated enzyme, *B.anthracis* cyclase.

To gain more insight into the specific role of Asp188 and Asp190 which resulted indeed to be essential for both catalysis and nucleotide-binding in *B.pertussis* enzyme, the ^{184}PLTADID^{190} region has been used to scan protein sequence libraries for enzymes having either nucleoside or/and carbohydrate phosphates as substrates and possessing the two adjacent aspartate residues in their catalytic center.

Table II. Interaction of modified forms of *B.pertussis* adenylate cyclase with CaM as determined from kinetic analysis and competitive binding experiments.

Enzyme	K_d for CaM (nM)
Wild-type	0.2[a]
D188N	0.7[b]
D188Y	2.1[b]
D190Y	2.2[b]
H298R	0.6[b]
H298L	3.2[b]
E301K	0.8[b]
E301Q	0.25[a]
T19	20[b]

[a]From activation curve of adenylate cyclase by various concentrations of CaM.
[b]From competitive binding experiments. The reaction was performed in medium containing 50 mM Tris–HCl (pH 8), 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 nM bovine brain CaM, 20 nM wild-type adenylate cyclase and 0–500 nM of modified forms of enzyme. The reaction was started with ATP (2 mM final concentration, 5×10^5 c.p.m./assay). The residual activity of the mutated adenylate cyclases was negligible even at their highest concentrations.

A prominent sequence thus extracted is that of phosphofructokinase whose crystal structure in its complex with reaction products has recently been described (Shirakihara and Evans, 1988). The sequence GIPLTADID in *B.pertussis* enzyme (which includes Asp188 and Asp190) is highly similar to the G^{V}_{L}PGTIDND sequence in the phosphofructokinase from *Bacillus stearothermophilus* or *E.coli*, respectively. Using the latter enzyme to scan libraries we found that it can also be aligned with several other ATP-binding enzymes such as pyruvate kinase, thymidylate kinase or DNA polymerase (Joyce *et al.*, 1982; Burke *et al.*, 1983; Jong *et al.*, 1984). It is, therefore, conceivable that Asp188 and Asp190 of *B.pertussis* adenylate cyclase interact with Mg^{2+}-ATP (most probably as a β, γ-bidentate complex) as accepting a hydrogen bond from a water ligand of Mg^{2+} or/and directly coordinating the Mg^{2+} through the carboxylate group (Figure 3). Participation of Asp188 and Asp190 in the catalytic step would consist in stabilizing the transition state. It is significant in this respect that changes in charge (Asp → Asn substitution) altered catalysis much more than nucleotide-binding properties, and that replacement of Asp188 and Asp190 by unrelated amino acids (His or Tyr) abolished completely catalysis.

It is of interest to recall here the role of another couple of neighbouring residues in *B.pertussis* adenylate cyclase, namely Lys58/Lys65, equivalent to Lys346/Lys353 in *B.anthracis* enzyme. These basic residues belong to the first segment of identity of the bacterial cyclases (Figure 1) being part (Lys65 and Lys353, respectively) of the consensus sequence GXXXXGKT(S). Their substitution by several other amino acid residues has also been shown to decrease by more than three orders of magnitude or to abolish the catalytic activity and to decrease by one or two orders of magnitude the nucleotide-binding properties of bacterial enzymes (Au *et al.*, 1989; Glaser *et al.*, 1989; H.Munier *et al.*, unpublished data). According to the current view concerning the role of similar Lys residues in other ATP-dependent enzymes, we assume that Lys65 (or Lys58) interacts with the α-phosphate group of Mg^{2+}-ATP, as shown in Figure 3. The validity of this model could be verified by using suitable analogues of pyrophosphate and cAMP in binding studies with the wild-type and modified forms of adenylate cyclase in which Lys58, Lys65, Asp188 or Asp190 have been substituted by other amino acids.

It is more difficult to assign a particular role to His298 and Glu301 in *B.pertussis* adenylate cyclase. Kinetic studies with bacterial or eukaryotic adenylate/guanylate cyclases using phosphorothionate analogues of ATP/GTP have suggested that the cyclization of these nucleotides takes place by a single nucleophilic displacement without formation of enzyme-nucleotidyl intermediates (Gerlt *et al.*, 1980; Eckstein *et al.*, 1981; Koch *et al.*, 1990). In the mechanism proposed by Gerlt *et al.* (1980), ionization of the 3′-OH group in ATP required participation of an amino acid side chain as a general basic catalyst. If the imidazole side chain of His298, which seems suited to such a role, is indeed involved in catalysis we expected that the His → Arg substitution would shift the optimum pH of *B.pertussis* adenylate cyclase to a more alkaline value. Determination of residual activity at pH 8 and 11 gave a ratio identical to that exhibited by the wild type enzyme. Moreover, in screening revertants of the above-mentioned mutation, we found that the H298N variant of adenylate cyclase is ~150 times more active than the 'parent' H298R mutant. The same was true for a revertant (E301N) resulting from the E301K mutation whose activity represented ~2% of that of wild-type enzyme. We suppose, therefore, that His298, as well as Glu301, are not involved directly in catalysis but participate in the activation of the bacterial enzyme by CaM. Interactive contacts, through hydrogen bondings, between these residues and segments of the polypeptide chain belonging to the active site of bacterial enzyme would optimize binding of the nucleotide substrate to the catalytic site and promote cyclization of ATP. Such 'activating' contacts occur or are facilitated upon binding of CaM to adenylate cyclase. Two arguments favour this hypothesis: (i) Gln successfully replaced Glu at position 301; and (ii) binding of Ant-dATP which occurs preferentially in the presence of CaM, was significantly affected in E301K, H298R and H298L mutants.

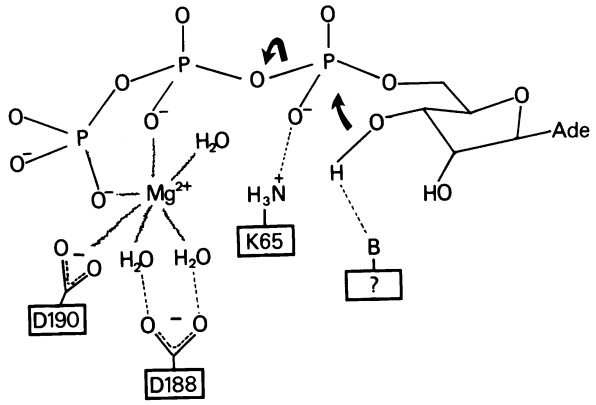

Fig. 3. Proposed interactive contacts between the polyphosphate chain of ATP and several amino acid side chains in *B.pertussis* adenylate cyclase. B is an as yet unidentified amino acid side chain which acts as a general basic catalyst in the cyclization of ATP.

Table III. Binding of [α-^{32}P]Ant-dATP to *B.pertussis* adenylate cyclase and to its modified forms as determined by equilibrium dialysis[a]

Enzyme	K_d (μM)
Wild-type	8
D188N	49
D188Y	≥300[b]
H298R	50
H298L	66
E301K	45

[a]Aliquots of 0.25 ml of a solution having a final concentration of 10–50 μM adenylate cyclase, CaM (in a 1.2 excess over enzyme), 50 mM Tris–HCl (pH 8), 100 mM NaCl, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, were equilibrated for 12 h with an equal volume of the same solution in which enzyme was replaced by radiolabelled Ant-dATP (≈2000 c.p.m./nmol) from 5 to 200 μM. Equilibrium data were analysed by Scatchard plots.

[b]Experimental points were too scattered for a precise determination of K_d. The K_d value was estimated assuming that the wild-type protein and D188Y mutant have identical binding stoichiometry.

Materials and methods

Chemicals

Adenine nucleotides, substrates, restriction enzymes, and T4 DNA ligase were obtained from Boehringer Mannheim. T7 DNA polymerase and the four deoxyribonucleoside triphosphates used in sequencing reactions were from Pharmacia. CaM-agarose, bovine brain CaM, dansyl-CaM, L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)–trypsin and soybean trypsin inhibitor were from Sigma. Urea (fluorimetrically pure)

was a product of Schwartz/Mann. [α-^{32}P]ATP (3000 Ci/mmol), [α-^{32}P]-dATP (1000 Ci/mmol), [α-^{35}S]ATP (1000 Ci/mmol), and [^{3}H]cAMP (40 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, UK. 3′-anthraniloyl-2′-deoxyadenosine 5′ triphosphate (Ant-dATP) was prepared from dATP and isatoic anhydride essentially by the same procedure described by Hiratsuka (1983) for the synthesis of Ant-ATP. The identity and the purity of the compound were checked by UV-spectrophotometry, mass spectrometry and ^{1}H-NMR. For synthesis of ^{32}P-labelled Ant-dATP, 50 μCi of [α-^{32}P]dATP plus 5 μmol of cold dATP were reacted with isatoic anhydride, then the product was purified by chromatography on Lichroprep RP-18 (25–40 μm) using 1 mM triethylammonium acetate as eluant (Sarfati *et al.*, 1990).

Bacterial strains and growth conditions

The *E.coli* BMH7118 (Gronenburn, 1976) strain was used for sequence analysis and site-directed mutagenesis. Production of recombinant protein was performed using the protease-deficient strain Y1083 BNN103 (Young and Davis, 1983) which habours plasmid pDIA5227 or a derivative and plasmid pDIA17. Plasmid pDIA5227 encodes a 432 residue long, truncated adenylate cyclase of *B.pertussis* (Gilles *et al.*, 1990). The 33 N-terminal amino acids of this protein numbered from −33 to −1 are encoded by vector sequences. They are followed by the first 399 amino acid residues of the adenylate cyclase (Figure 1). Plasmid pDIA17 expresses the *lacI* gene inserted in the tetracycline gene of plasmid pACYC184 in such a way as to be, at least in part, under the control of the tetracyclin promoter. Cultures were grown in LB medium (Miller, 1972) supplemented with 100 μg/ml ampicillin and 20 μg/ml chloramphenicol. Synthesis of truncated adenylate cyclase was induced by IPTG (1 mM) when cultures reached an optical density of 0.5. Bacteria were harvested by centrifugation, 4 h after induction.

Site-directed mutagenesis and sequence analysis

Oligonucleotide-directed mutagenesis was performed on the single-stranded form of pDIA5227 using the Amersham system based on the method of Taylor *et al.* (1985). Sequences of oligonucleotides used are indicated in Table I. For each mutagenesis the whole sequence of the *cya* gene was controlled for the absence of any other mutations by the dideoxynucleotide sequencing method (Sanger *et al.*, 1977).

Purification and assay of adenylate cyclase

Truncated *B.pertussis* adenylate cyclase expressed in *E.coli* was purified by affinity chromatography on CaM-agarose, essentially as described by Glaser *et al.* (1989). T19 peptide, corresponding to the C-terminal domain of adenylate cyclase (residues 225–399) was prepared and purified as previously described by Gilles *et al.* (1990). Enzyme activity was measured using the procedure of White (1974) as described previously (Ladant, 1988; Ladant *et al.*, 1989). One unit of adenylate cyclase activity corresponds to 1 μmol cAMP formed in 1 min at 30°C and at pH 8.

Fluorescence measurements

Binding of adenylate cyclase to dansyl-CaM or to Ant-dATP was analysed with a Perkin–Elmer LS-5B luminescence spectrometer maintained at 25°C using 1 × 1 cm or 1 × 0.2 cm UV-grade quartz cuvettes (sample volume of 2 or 0.3 ml). The dansyl moiety of CaM was excited at 340 nm and Ant-dATP was excited at 330 nm. The titration of fluorescence enhancement or quenching was performed by recording the fluorescence emission at 480 nm (dansyl-CaM) or 420 nm (Ant-dATP). One data point corresponds to fluorescence intensities integrated over a total time of 8 s.

The intrinsic fluorescence spectra (λ_{exc} = 295 nm) of wild-type and mutant enzymes (1 μM) in the absence or presence of CaM (1.2 μM) were recorded in 50 mM Tris–HCl (pH 8), 0.1 M NaCl and 0.1 mM $CaCl_2$ from 305 to 400 nm.

Analytical procedures

Protein concentration was determined according to Bradford (1976) using a BioRad kit or according to the procedure of Smith *et al.* (1985) as purchased from Pierce Chemical Co. SDS–PAGE was performed as described by Laemmli (1970). Binding of [α-^{32}P]Ant-dATP to adenylate cyclase by equilibrium dialysis was determined at 6°C in chambers of 0.5 ml capacity as previously described (Sarfati *et al.*, 1990). The dissociation constant of CaM in complexes with inactive forms of adenylate cyclase was determined by competition with wild-type active protein (see Appendix).

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Appendix

Determination of small dissociation constants ($K_d < 10^{-9}$ M) of calmodulin in complexes with various peptides by the competition method

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One difficulty frequently encountered in the analysis of peptides derived from *B.pertussis* adenylate cyclase or of inactive forms of the enzyme was the accurate determination of dissociation constants (K_d) of their complexes with CaM. Spectroscopic techniques, including fluorescence analysis, are suitable for analysis of K_d values higher than 10^{-8} M. As *B.pertussis* adenylate cyclase has a K_d value for CaM close to 10^{-10} M, we propose a new competitive method to determine in a single experiment the dissociation constant of a peptide–CaM complex in this same range of K_d values.

The system involves the following binding equilibria: E + C ⟷ CE and P + C ⟷ CP, where E stands for adenylate cyclase, C for CaM, P for peptide and CE and CP for the complexes of CaM with the enzyme and with the peptide, respectively.

The equilibrium of the system may be described in terms of the following five equations, of which (1) and (2) define the macroscopic dissociation constants K_d (for the CaM–enzyme complex) and K'_d (for the CaM–peptide complex), while (3)–(5) express conservation laws (with subscript *f* denoting concentration of free component at equilibrium and subscript *i*, initial concentration):

$$K_d = C_f \times E_f/CE \quad (1)$$

$$K'_d = C_f \times P_f/CP \quad (2)$$

$$C_i = C_f + CE + CP \quad (3)$$

$$P_i = P_f + CP \quad (4)$$

$$E_i = E_f + CE \quad (5)$$

The following relationship may be then obtained by dividing equation (1) by equation (2) and using P_f from equation (4) and E_f from equation (5):

$$\frac{K_d}{K'_d} = \frac{(E_i - CE)}{(P_i - CP)} \times \frac{CP}{CE} \quad (6)$$

At the same time, one may introduce a useful new variable by dividing the top and bottom of equation (6) with C_i, defined as:

$$x = CE/C_i \quad (7)$$

which represents the fraction of CaM that is complexed with enzyme. With this notation and using equation (3), the fraction CP/CE, which appears in the right hand side of equation (6) may be written as $(1-x-C_f/C_i)/x$. To bring this equation to a more practical form, one should bear in mind that one is dealing with small dissociation constants and that the experimental conditions are such that the enzyme and the peptide are in excess over CaM ($C_f \ll C_i$, $CP \ll P_i$, $CE \ll E_i$). Thus, after the elimination of the negligible terms, equation (6) becomes:

$$\frac{K_d}{K'_d} = \frac{E_i}{P_i} \times \frac{(1 - x)}{x} \quad (8)$$

which relates the dissociation constants to the known initial concentrations of enzyme and peptide and the measurable variable *x*.

In the case of the adenylate cyclase system, *x* may be determined experimentally as the ratio of enzyme activities in the presence and in the absence of the peptide inhibitor.

The six simultaneous equations (1), (7), (8) and (3)–(5), which contain a total of 11 parameters, may be solved for six unknowns, chosen to suit the actual situation. For the purpose of testing the validity of the practical relationship (8) by a numerical application, but also in the planning phases of competition experiments, one may consider as given the two dissociation constants and the *initial* concentrations, and then solve the simultaneous equations for the expected value of parameter *x*, and the equilibrium concentrations. One may further recalculate the dissociation constants, based on their definitions, using the calculated values of the *equilibrium* concentrations of the components. One obtains:

$$x = \frac{K'_d \times E_i}{K_d \times P_i + K'_d \times E_i} \quad (9)$$

$$CP = \frac{E_i \times C_i - x \times (C_i^2 + E_i \times C_i + K_d \times C_i) + x^2 \times C_i^2}{E_i - x \times C_i} \quad (10)$$

$$CE = x \times C_i \quad (11)$$

$$E_f = E_i - CE \quad (12)$$

$$P_f = P_i - CI \quad (13)$$

$$C_f = C_i - CE - CP \quad (14)$$

The following table illustrates this application. The small (9%) discrepancy between the actual and the recalculated values of K'_d comes from the use of the practical form, (9) [identical to (8)], of equation (6) in the simultaneous equations, and from the presence of the very small quantity C_f in equation (2).

Table. Numerical data used to assess the validity of the theoretical model.

Parameter	Initial data	Calculated data	Recalculated values, using calculated data
K_d (nM)[a]	0.2	–	0.20024[c]
K'_d (nM)[a]	10	–	10.869[d]
E_i (nM)	20	–	–
C_i (nM)	1	–	–
P_i (nM)	300	–	–
x	–	0.7692308[b]	0.7562311[e]
CE (nM)	–	0.7692308	–
CP (nM)	–	0.2227692	–
E_f (nM)	–	19.23077	–
P_f (nM)	–	299.7772	–
C_f (nM)	–	0.008000	–

[a]Data obtained from the activation curve for *B.pertussis* adenylate cyclase by CaM. K_d of 10 nM was arbitrarily chosen.
[b]Based on equation (8).
[c]Equation (1).
[d]Equation (2).
[e]Based on equation (6).

At the same time, the discrepancy between the value of *x* obtained using equation (9) and that recalculated using the unabridged form (6), which requires knowledge of the equilibrium concentrations, is as small as 1.7%. The smallness of this quantity is mainly due to the choice of the experimental conditions, i.e. excess E and P over C, and also reflects the fact that the relative errors conferred upon P_i and E_i, by neglecting CP and CE, respectively, from equation (6), both have the same sign, a situation in which the fraction suffers least. Thus, for any practical use, relationship (6) is entirely satisfactory.